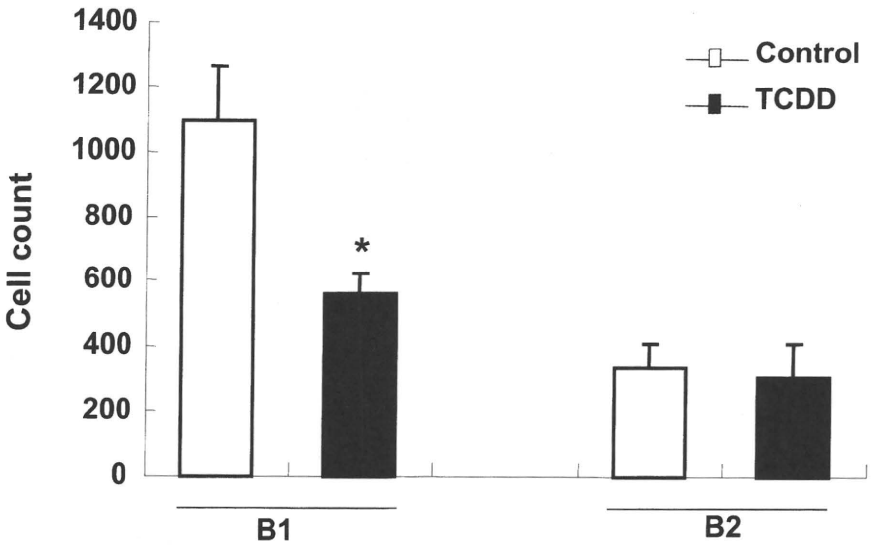

Fig. 10. Impaired chemotaxis of B1, but not B2 cells TCDD-treated mice. B1 and B2 cells enriched by MACS® beads were subjected to chemotaxis assay on BLC/cXCL13 as described in Materials and Methods. * $p < 0.001$.

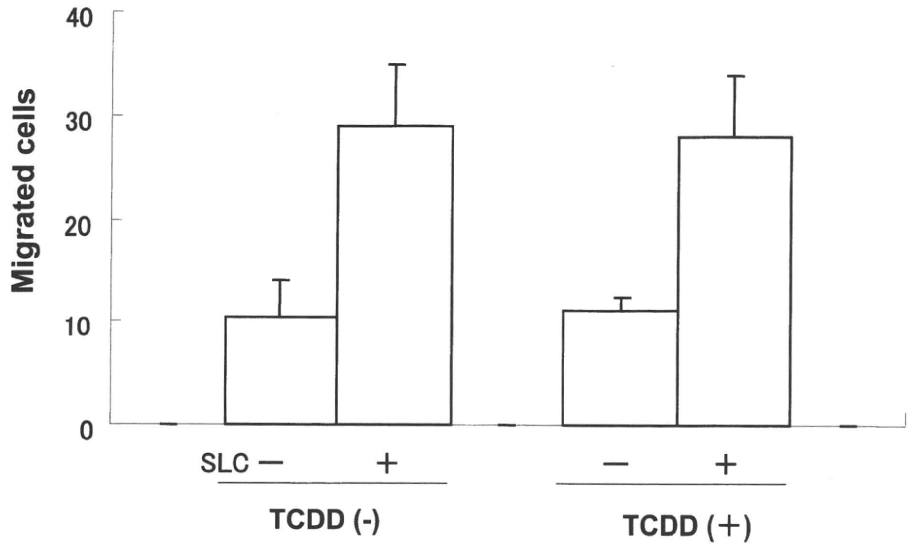

Fig. 11. Chemotaxis assay on dendritic cells (DCs). $CD11c^+$ cells obtained from mesenteric LNs and PPs of TCDD-treated or control mice by MACS® beads were subjected to chemotaxis assay on SLC/CCL21 as in Fig. 9.

ticular chemokine receptors were decreased in B1-cells, a unique B-cell subset which is distinguished from conventional B-cells (B2 cells) by their origin, cell surface phenotype and unique tissue distribution, capacity for self-renewal (Hardy and Hayakawa, 1986). B1-cells have been considered to be involved in autoantibody production in the development of particular autoimmune diseases. It is also reported that approximately half of IgA^+ cells in the intestinal lamina propria are derived from B1-cells and that they play a pivotal role for innate mucosal immunity in the gut although the site for IgA class-switching and differentiation to IgA-secreting plasma cells remains

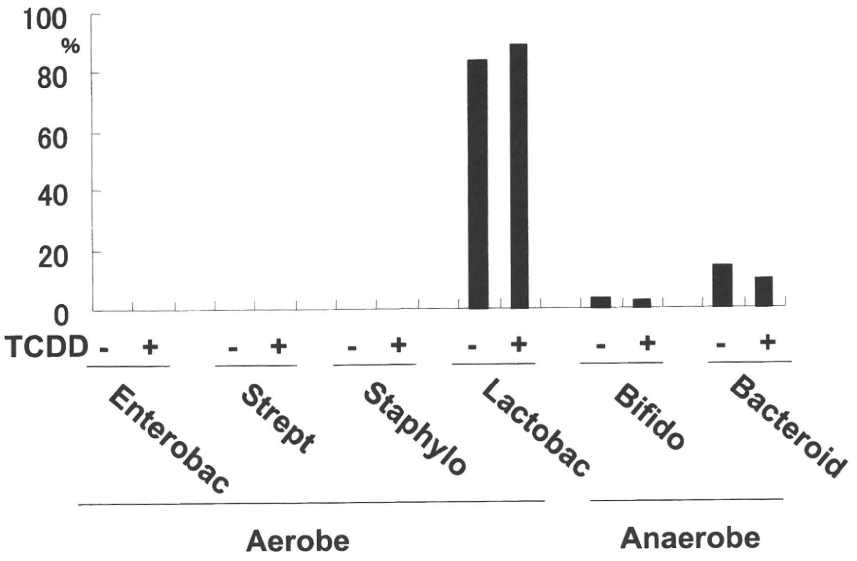

Fig. 12. Effect of TCDD on the number and frequency of commensal microflora in the intestine. Fecal samples were collected one week after TCDD administration and the number and frequency of aerobes (Lactobacillus, Enterococcus, Streptococcus, Staphylococcus) and anaerobes (Bacteroides and Bifidobacterium) were determined after appropriate culture at Intestinal flora laboratory of Calpis Co., Ltd..

to be elucidated (Kroese *et al.*, 1989; Beagley *et al.*, 1995; Murakami and Honjo, 1995; Macpherson *et al.*, 2000). It has been recently demonstrated that constitutively active aryl hydrocarbon receptor causes selective loss of peritoneal B1a ($CD5^{+}B220^{low}IgM^{high}$) cells (Anderson *et al.*, 2003), suggesting that the B1-cell is a sensitive cellular target for TCDD. On the other hand, ex vivo IgM secretion by B1-cells on LPS simulation was not affected by TCDD treatment as shown in the present study, suggesting that decreased level of IgA in the gut was not attributed to direct effect of TCDD on Ig production. Instead, we favor the idea that impaired B1-cell trafficking by TCDD from the peritoneal cavity to the intestinal mucosa could be one of the causes for decreased level of IgA in the gut (Fig. 13).

Another important finding in the present study is that TCDD exposure by breast feeding also results in decreased level of intestinal IgA in the pups. Maternal depots of TCDD, stored primarily in adipose tissue, are efficiently transferred to pups during nursing period. Maternal TCDD levels rapidly decreased during lactation period while tissue levels in the nursing pups increased. Tissue levels in the offspring even exceed those of their mothers during the 3-week period after birth. Thus, lactation serves as a significant route of exposure for the developing neonate (Nau *et al.*, 1986). It is previously reported that lactational exposure to TCDD induced hydronephrosis both in mice and rats (Couture-Haws *et al.*, 1991; Nishimura *et al.*, 2006). Since development of mucosal immune system does not cease at birth as well as renal development, it is not surprising that TCDD exposure by breast milk decreases IgA levels in the gut at relatively low dose. Furthermore, immature mucosal immune system of newborn infants may be much more sensitive to TCDD exposure. Therefore, our findings would have a significant impact on the risk assessment of TCDD for neonates and infants given that babies with breast-feeding take 15-20 times more TCDD than the dose of tolerable daily intake (TDI). Decreased level of IgA in the gut would result in increased frequency of infection and altered distribution of commensal bacterial microflora which plays a role for oral tolerance.

Breakdown of oral tolerance by TCDD is another interesting finding in the present study. Oral tolerance is historically and originally described as antigen-specific inhibition of systemic IgG antibody production by oral pre-administration of protein antigen (Strobel and Mowat, 1998; Faria and Weiner, 1999). Many studies have been performed using a similar experimental protocol to that used in the present study to demonstrate the presence or absence of oral tolerance. However, the precise mechanism for oral tolerance still remains controversial

Impaired mucosal immunity in the gut by TCDD

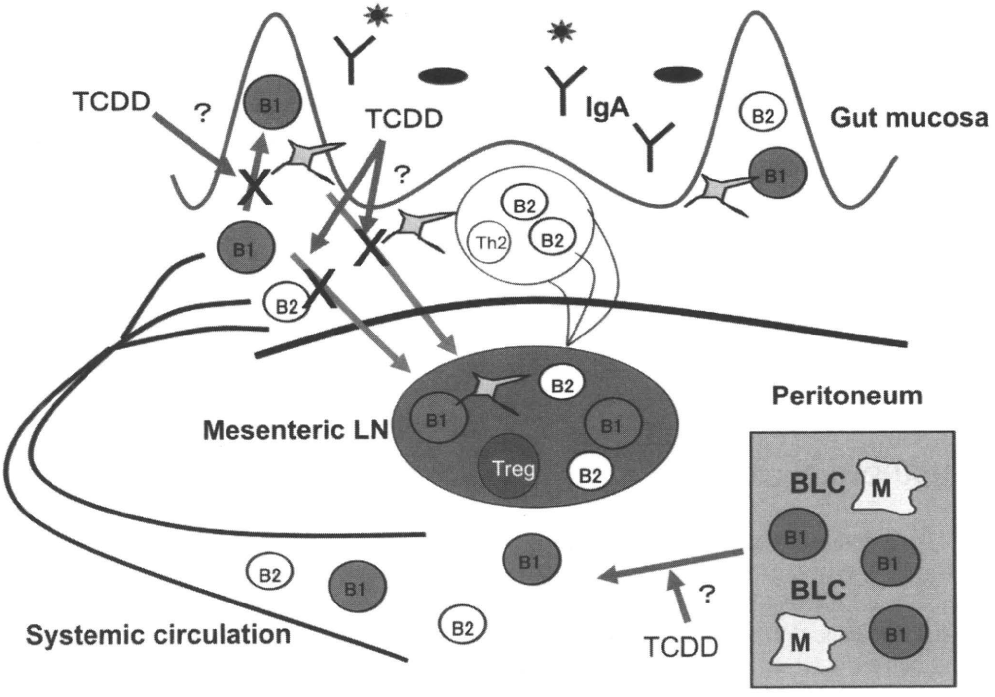

Fig. 13. Hypothetical model for TCDD-induced breakdown of mucosal immunity. TCDD treatment possibly impaires B1-cell trafficking from the peritoneal cavity to the lamina propria in gut. This results in decreased level of IgA in the gut lumen without directly affecting Ig secretion.

(Mowat, 2003). It was previously reported that regulatory T-cells producing TGF-β and/or IL-10 were induced in PPs upon oral administration of protein antigens (Santos *et al.*, 1994; Tsuji *et al.*, 2001). However, the classical idea for the pivotal role of PPs in oral tolerance has been challenged by several studies demonstrating that oral tolerance could be induced independently of PPs (Spahn *et al.*, 2001). It was also demonstrated that the spleen plays an important role for oral tolerance (Suh *et al.*, 1993). These results favor the idea that mesenteric LNs and/or spleen are critical lymphoid organs as the induction site for oral tolerance although they do not exclude a physiological role of PPs. Accumulating evidence also suggests that DCs in the gut play a pivotal role for oral tolerance (Viney *et al.*, 1998, Huang *et al.*, 2000; Chirdo *et al.*, 2000, Sun *et al.*, 2007). However, the frequency of $CD11c^+$ cells remained unchanged in PPs, mesenteric LNs and spleen before and after TCDD treatment. Functional analysis on DCs with different localization is under investigation to elucidate the mechanism involved in disrupted oral tolerance by TCDD. It has been recently demonstrated that TCDD generates $CD25^+CD4$ T cells with regulatory function in a GVH model (Funatake *et al.*, 2005). However, FACS analysis showed only a marginal change in the frequency of $CD25^+CD4$ T cells in the PPs, mesenteric LNs, and spleen (6.3 ± 0.28, 12.3 ± 0.28, 8.95 ± 0.21 in TCDD-treated mice and 4.25 ± 0.35, 11.4 ± 0.21, 7.6 ± 0.28 in control mice, respectively). Effect of TCDD on Treg trafficking remains to be examined. Although commensal bacterial microflora plays a role in the development of oral tolerance (Sudo *et al.*, 1997), there was no change in the number and frequency of major bacterial species.

As a result of breakdown of oral tolerance, T-cells in PPs, axillar inguinal and cervical LNs were sensitized by orally administered OVA. It is known that patients with atopic dermatitis show high frequency of food allergy and that dietary allergens such as egg albumin often turn out to be the allergen in the skin in these patients, indicating the existence of immunological cross talk between the intestinal mucosa and the skin. It is considered that microbial infection, excessive antibiotic administration, early onset of a weaning diet and so on are the causes for systemic allergic sensitization to oral antigens in infants.

Our findings suggest that TCDD can be a possible candidate for such disruptors for mucosal immunity leading to allergic sensitization.

Collectively, we have demonstrated that relatively low dose of TCDD results in breakdown of intestinal mucosal immunity and systemic sensitization by oral antigen in mice. Disrupted chemotaxis of B1 cells may be one of the mechanisms for defective IgA in the gut in TCDD-treated mice. Thus, immunological effects of environmental chemicals such as dioxins should be assessed on the basis of mucosal immunity in the gut. This may also provide a new insight for understanding environmental factors responsible for increased allergic diseases in recent decades.

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Immunopathology and Infectious Diseases

Increased Foxp3$^+$ CD4$^+$ Regulatory T Cells with Intact Suppressive Activity but Altered Cellular Localization in Murine Lupus

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Foxp3$^+$ CD4$^+$ regulatory T (T_{reg}) cells play a pivotal role in the maintenance of dominant self tolerance. Understanding how the failures of immune control by T_{reg} cells are involved in autoimmune diseases is important for the development of effective immunotherapies. In the present study, we analyzed the characteristics of endogenous T_{reg} cells in (NZB × NZW) F1 (BWF1) mice, a murine model of systemic lupus erythematosus. Unexpectedly, T_{reg} number and frequency in aged BWF1 mice with developing lupus nephritis were increased, not decreased, and *in vitro* suppressive activity in lymphoid organs was intact. In addition, T_{reg} cells trafficked to target organs because cells were present in the kidney and lung. T_{reg} cells of aged BWF1 mice exhibited altered localization within lymph organs, however, and an altered phenotype, with higher expression levels of chemokine receptors and activation markers, suggesting a highly activated cellular state. Notably, the expression levels of costimulatory molecules were also markedly enhanced in the T_{reg} cells of aged BWF1 mice. Furthermore, T_{reg} cells of BWF1 mice did not show any suppressive effects on antibody production *in vitro*. Taken together, we conclude that T_{reg} cells in BWF1 mice are not predisposed to functional incompetence but rather are present in a highly activated state. *(Am J Pathol 2008, 173:1682–1692; DOI: 10.2353/ajpath.2008.080314)*

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology characterized by a massive production of autoantibodies against various nuclear antigens. The deposit of immune complexes in the target organs, ie, skin, kidney, lung, joints, and central nervous system, is thought to cause fatal dysfunction of the body system. (NZB × NZW) F1 (BWF1) is a mouse strain that has been widely used as a model for SLE since the 1960s. These mice spontaneously develop severe autoimmune disease highly resembling human SLE in terms of serological and hematological abnormalities, and severe nephritis accompanying massive production of antinuclear antibodies.[1]

Reconstitution of SCID (severe combined immunodeficiency) mice with cultured pre-B cells of BWF1 mice recapitulates many symptoms of the disease of BWF1 mice. Cultured pre-B cells alone, however, are not sufficient to fully reproduce the disease.[2] These data suggest that cellular subset(s) in addition to B cells are necessary for the development of the lupus-like syndrome of BWF1 mice, although abnormalities of the immune system predominantly lie within B cells. One of the possible candidates is CD4$^+$ T cells, because depletion of CD4$^+$ T cells with anti-CD4 antibody from 5 months old, slightly before the disease onset, prevents the development of the disease.[3,4] CD4$^+$ T cells are, therefore, also required for the development of the disease in BWF1 mice, possibly by providing help for the production of high-affinity autoantibodies.

Studies in this decade have clearly shown the key roles of naturally occurring regulatory T (T_{reg}) cells in the maintenance of dominant self tolerance of the immune system.[5] T_{reg} cells in normal mice are mostly of thymic origin and are considered to be autoreactive T-cell clones that have bypassed negative selection by unknown mechanism(s).[6] There also exists T_{reg} cells of extra-thymic origin induced from conventional T cells during immune responses,[7] although the underlying mechanisms of this

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process are still unclear. Foxp3, a member of forkhead-box family of transcription factors, is specifically expressed in the whole life of T_{reg} cells and programs their functional properties.[8–10] In contrast to the previously used marker CD25 or combination of CD25 and CD62L, expression of Foxp3 is specific for T_{reg} cells, and thus can be used for the definitive identification of these cells.[11] Immunoregulatory function of T_{reg} cells is dependent on Foxp3 and genetic deficiency of *Foxp3* causes fatal organ-specific autoimmune disease because of the lack of functional T_{reg} cells.[12–14] Furthermore, many groups have reported the reduced number and/or function of T_{reg} cells in both organ-specific and systemic autoimmune diseases.[15]

A recent study has shown that the decreased frequency of T_{reg} cells in the peripheral blood was associated with disease activity in SLE patients.[16] Frequency of T_{reg} cells identified as $CD25^+$ $CD62L^{hi}$ $CD4^+$ T cells in the spleen was also decreased in aged BWF1 mice.[17] Accordingly, adoptive transfer of *in vitro*-expanded T_{reg} cells, or administration of histone-derived peptides or peptides derived from the complementarity-determining region 1 of anti-double-strand DNA immunoglobulin has been shown to ameliorate the disease in BWF1 mice by a mechanism involving T_{reg} cells.[17–20] These studies suggest that the function of endogenous T_{reg} cells is, at least partially, abrogated by unidentified mechanisms in BWF1 mice.

Despite the effort to develop therapeutic methods involving T_{reg} cells, their nature in BWF1 mice remains unclear. Here we performed a detailed characterization of T_{reg} cells in BWF1 mice using Foxp3 as their marker. Our results demonstrated that aged BWF1 mice had increased frequency and number of T_{reg} cells with apparently normal function, but with an activated phenotype including enhanced expression of co-stimulatory molecules and altered localization.

Materials and Methods

Mice

Female 6- to 8-week-old BWF1 and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) and were kept under specific pathogen-free conditions in the animal facility of our laboratory until analysis. Mice were used at 6 to 10 or 32 to 40 weeks of age as young or aged, respectively. All animal experiments were approved by the animal care committee of The University of Tokyo.

Antibodies

Monoclonal anti-mouse CD4 (clone RM4-5), CD5 (55-7.3), CD8α (53-6.7), CD11b (M1/70), CD16/32 (2.4G2), CD19 (1D3), CD23 (B3B4), CD25 (7D4), CD43 (S7), CD44 (IM7), CD45 (30-F11), CD45R (RA3-6B2), CD62L (MEL-14), CD69 (H1.2F3), CD90.2 (53-2.1), CD103 (M290), OX40 (OX-86), CXCR4 (2B11/CXCR4), CCR5 (C34-3448), NK1.1 (PK136), TER-119 (TER-119), and streptavidin were purchased from BD Biosciences (San Diego, CA); monoclonal anti-mouse 4-1BB (17B5), ICOS (7E.17G9), F4/80 (BM8), CCR7 (4B12), and Foxp3 (FJK-16s) were purchased from eBioscience (San Diego, CA); monoclonal anti-mouse CXCR3 (220803) was purchased from R&D Systems (Minneapolis, MN). Antiserum raised against mouse type IV collagen was purchased from LSL (Tokyo, Japan). Details of monoclonal anti-mouse CCR4 antibody (clone 2G11) will be described elsewhere by Nagakubo and colleagues.[21,22]

Cell Isolation

Single cell suspensions of the thymus, spleen, and lymph nodes were prepared by passing the tissue through a cell strainer (BD Bioscience). Single cell suspension of the kidney and lung were prepared by dissociating the tissue with collagenase D (Roche, Basel, Switzerland). Mononuclear cells in the kidney and lung were isolated from the single cell suspension by Percoll (Invitrogen, Carlsbad, CA) gradient centrifugation. $CD25^+$ $CD4^+$ T cells were isolated from the single cell suspension of various organs by magnetic enrichment of $CD25^+$ cells followed by fluorescence-activated cell sorting with the Epics Altra cell sorter (Beckman Coulter, Fullerton, CA). $CD25^-$ $CD4^+$ T cells were isolated from the single cell suspension of spleen by magnetic depletion of the cells positive for CD8α, CD11b, CD25, B220, CD138, NK1.1, or TER-119. B1 cells were isolated from peritoneal lavage cells by magnetic depletion of the cells positive for CD23, Thy-1.2, or F4/80. B2 cells were isolated from spleen by magnetic depletion of the cells positive for CD43, Thy-1.2, or TER-119. All procedures involving magnetic isolation were performed with an autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow Cytometry

Cells were incubated with fluorochrome- or biotin-labeled antibodies for 20 minutes at 4°C, following the blockade of FcγRII/III with unlabeled anti-CD16/32 for 10 minutes at 4°C; for the staining with biotin-labeled anti-CCR7, incubation after the blockade of Fc receptors was performed at 37°C. Biotin-labeled antibodies were visualized by further incubating with phycoerythrin-conjugated streptavidin for 15 minutes at 4°C. Staining of Foxp3 was performed according to the manufacturer's instructions. Data were collected using BD LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunofluorescent Staining

Explanted tissues embedded in OCT compound were snap-frozen in liquid nitrogen and stored at −80°C until use. Six-μm-thick sections of frozen tissues were fixed with cold acetone for 10 minutes and rehydrated with phosphate-buffered saline (PBS) for 10 minutes at room temperature. Rehydrated sections were blocked for nonspecific binding of proteins with Blocking One (Nacalai Tesque, Kyoto, Japan) for 20 minutes at room temperature and incubated with unlabeled or biotinylated anti-

Table 1. Primers and Probes for Real-Time PCR

Gene	Sense	Probe	Antisense
CCL19	5′-GAAAGCCTTCCGCTACCTTCT-3′	5′-CCCATCCCGGCAATCCTGTTCTTA-3′	5′-CCCTTAGTGTGGTGAACACAA-CA-3′
CCL21	5′-GGCTATAGGAAGCAAGAACCAA-GT-3′	5′-TTACTTCTACCGACGTCCCACGGA-3′	5′-TCAGGCTTAGAGTGCTTCCG-3′
CXCL9	5′-TGATAAGGAATGCACGATGCTC-3′	5′-AGCCGAGGCACGATCCACTACAAA-TC-3′	5′-TTCCTTGAACGACGACGACTTT-3′
CXCL10	5′-CGTCATTTTCTGCCTCATCCT-3′	5′-AAGCTTGAAATCATCCCTGCGAG-CC-3′	5′-TGGTCTTAGATTCCGGATTCAG-3′
CXCL12	5′-GCTCTGCATCAGTGACGGTAA-3′	5′-ATCGCCAGAGCCAACGTCAAGCAT-CT-3′	5′-AGCCGTGCAACAATCTGAAG-3′
GAPDH	5′-AGTATGACTCCACTCACGGCAA-3′	5′-AACGGCACAGTCAAGGCCGAGAAT-3′	5′-TCTCGCTCCTGGAAGATGGT-3′

bodies, or antisera for 60 minutes at room temperature. Sections were then incubated with Alexa Fluor-labeled anti-Ig secondary antibodies or streptavidin (Invitrogen) for 30 minutes at room temperature. After the staining, sections were fixed with phosphate-buffered 4% paraformaldehyde for 10 minutes at room temperature and were mounted with Prolong Gold Antifade Reagent (Invitrogen). Specimens were observed under IX70 confocal laser-scanning microscopy (Olympus, Tokyo, Japan).

Quantification of Histological Analysis

Images obtained from confocal microscopic observation were processed with Win ROOF software (Mitani Corporation, Fukui, Japan), and the number of the signals was counted manually or automatically using Win ROOF software.

In Vitro *Proliferation and Suppression Assay*

2×10^4 cells/well of purified $CD25^-$ $CD4^+$ T cells were stimulated with 2 μg/ml of concanavalin A (Sigma-Aldrich, St. Louis, MO) and 8×10^4 cells/well of mitomycin-C (Sigma-Aldrich)-treated $Thy1.2^-$ splenocytes with or without titrated number of $CD25^+$ $CD4^+$ T cells were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mmol/L HEPES, 55 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin in a round-bottom 96-well plate for 72 hours at 37°C. $CD25^+$ $CD4^+$ T cells were cultured under the same conditions to measure their proliferative capacity in the absence of $CD25^-$ $CD4^+$ T cells. Cells were pulsed with 1 μCi/well [^{3}H-methyl]-thymidine (GE Health Care, Buckinghamshire, UK) for the last 6 to 8 hours of the culture, and proliferation was measured by cpm value of the harvested cells. Suppressive activity of $CD25^+$ $CD4^+$ T cells was expressed as percent suppression[23] calculated as following: 100 × [cpm (responder) − cpm($CD25^+$ + $CD25^-$)]/cpm(responder).

In Vitro *Antibody Production Assay*

In vitro antibody production by B cells was analyzed as previously described[24] with several modifications. Briefly, 2×10^5 B1 or B2 cells isolated from young or aged BWF1 mice and equal numbers of $CD25^-$ $CD4^+$ T cells isolated from the spleen of young or aged BWF1 mice were cultured with or without 1×10^5 $CD25^+$ $CD4^+$ T cells in supplemented RPMI 1640 medium for 5 days at 37°C. The concentration of IgG antibody in the culture supernatant was measured by enzyme-linked immunosorbent assay using a Mouse IgG quantitation kit (Bethyl, Montgomery, TX).

Preparation of cDNA and Real-Time Polymerase Chain Reaction (PCR)

Mice were perfused with 30 mL of PBS, and spleen, lymph nodes, kidney, and lung were excised. Tissues were homogenated with TRIzol reagent (Invitrogen), and purified total RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed with an ABI Prism 7500 (Applied Biosystems) using primers and Taq Man probes listed in Table 1.

Statistical Analysis

Statistical significance of the difference between data sets was analyzed by Welch's unpaired *t*-test for the comparison of two groups or by one-way analysis of variance with Bonferroni's multiple comparison test for more than three groups. $P < 0.05$ was considered to be statistically significant.

Results

Increased Number and Frequency of T_{reg} Cells in Aged BWF1 Mice

Suppressive activity of T_{reg} cells is strongly correlated with the expression of Foxp3.[11] To clarify whether an increase or decrease in the frequency and/or number of T_{reg} cells exists, we analyzed the population of $Foxp3^+$ $CD4^+$ T cells by flow cytometry. We found that aged BWF1 mice had substantially increased frequency (Figure 1A) and number (Figure 1B) of $Foxp3^+$ $CD4^+$ T cells in the lymphoid organs compared with young BWF1 mice. A recent study has shown an age-dependent increase in $CD25^-$ $Foxp3^+$ $CD4^+$ T cells in 24-month-old normal mice,[25] but increased $Foxp3^+$ $CD4^+$ T cells in aged BWF1 mice was not merely an age-dependent event be-

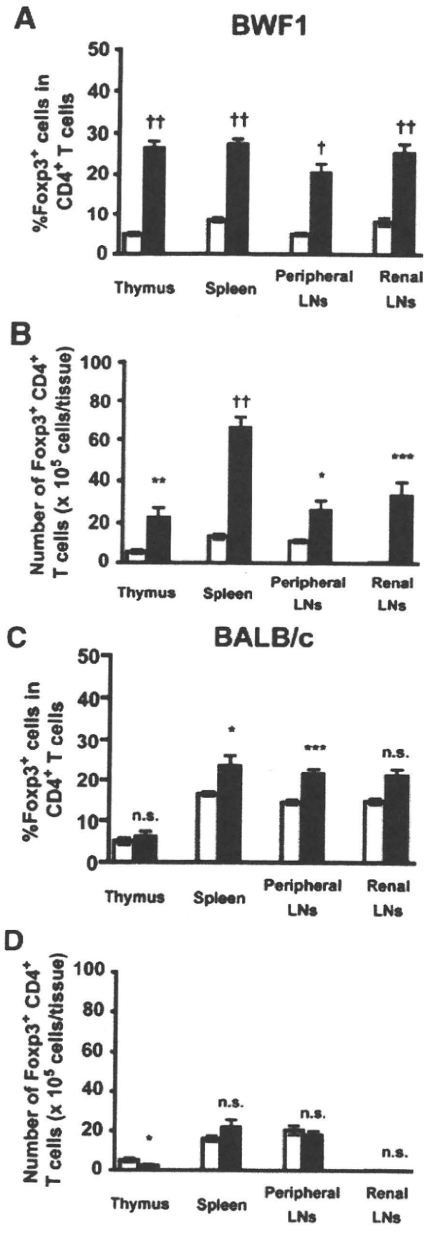

Figure 1. Increased Foxp3$^+$ CD4$^+$ T_{reg} cells in aged BWF1 mice. Frequency (**A**, **C**) and number (**B**, **D**) of Foxp3$^+$ CD4$^+$ T cells within thymus, spleen, peripheral LNs (inguinal, axillary, brachial, submandibular, and cervical), and renal lymph node of BWF1 (**A**, **B**) or BALB/c (**C**, **D**) mice were analyzed by flow cytometry. Data were presented as mean ± SEM (n = 4 to 9 for each group). Open bar, young; filled bar, aged. Statistical significance of the difference between young and aged mice was analyzed by Welch's unpaired t-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{\dagger}P < 0.0005$, $^{\dagger\dagger}P < 0.0001$.

cause age-matched BALB/c mice did not show a marked increase in Foxp3$^+$ CD4$^+$ T cells (Figure 1, C and D).

CD25$^+$ CD4$^+$ T Cells Showed Normal Suppressive Activity Both in Young and Aged BWF1 Mice

Valencia and colleagues[16] reported a decreased suppressive activity of CD25$^+$ CD4$^+$ T cells in SLE patients, possibly because of the lower proportion of Foxp3$^+$ cells among CD25$^+$ CD4$^+$ T cells. This result, however, does not exclude the possibility that a functional defect intrinsic to T_{reg} cells exists as well. To test the functional competency of T_{reg} cells of BWF1 mice, we performed an *in vitro* suppression assay. Because Foxp3 expression could be detected only in permeabilized cells, we used CD25$^+$ CD4$^+$ T cells as a surrogate for Foxp3$^+$ CD4$^+$ T cells. Concurrent with the high proportion of Foxp3$^+$ cells among CD25$^+$ CD4$^+$ T cells even after disease onset (Figure 2A), CD25$^+$ CD4$^+$ T cells isolated from the spleen and lymph nodes of both young and aged BWF1 mice did not proliferate on stimulation (Figure 2B) and showed suppressive activity (Figure 2C). Furthermore, CD25$^+$ CD4$^+$ T cells isolated from the kidney (Figure 2C) and lung (data not shown), ie, the target organs, of aged BWF1 mice also showed suppressive activity comparable to those from the spleen and lymph nodes. CD25$^+$ CD4$^+$ T cells of thymus or lymph nodes showed similar suppressive activity (data not shown). We did not note a significant difference in the suppressive activity between young and aged, or lymphoid and nonlymphoid CD25$^+$ CD4$^+$ T cells in BWF1 mice at any dose of CD25$^+$ CD4$^+$ T cells. Taken together, our data suggest that aged BWF1 mice have an expanded pool size of T_{reg} cells with intact suppressive activity.

T_{reg} Cells Infiltrated into the Target Organs

Defective migration into the site of inflammation is known to impair the *in vivo* suppressive activity of T_{reg} cells even if they were functionally competent *in vitro*.[26] Because our data indicated that T_{reg} cells of BWF1 mice have intact suppressive activity *in vitro*, we asked whether T_{reg} cells in aged BWF1 mice infiltrated into the target organs, ie, kidney and lung. Flow cytometric analysis of mononuclear cells within the target organs revealed that Foxp3$^+$ as well as Foxp3$^-$ CD4$^+$ T cells infiltrated into these organs, and that the frequency of Foxp3$^+$ cells in CD4$^+$ T cells was comparable to that in the lymph nodes of normal mice[11] (18.76 ± 3.79% in the kidney and 14.08 ± 2.50% in the lung). Foxp3$^+$ CD4$^+$ T cells infiltrated into the glomeruli, interstitium, and perivascular region of the kidney along with Foxp3$^-$ CD4$^+$ T cells (Figure 3B). Young BWF1 mice and nonautoimmune control mice did not show the infiltration of inflammatory cells (data not shown). Moreover, both Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells were apparently distributed evenly within the infiltrating site of the target organs (Figure 3, A and C), indicating that clustering of these cells that were essential for T_{reg} cell-mediated suppression[26,27] would take place in the target organs as well as in the lymphoid organs.

Medullary Localization of T_{reg} Cells within the Thymus

The thymus, another target organ of the disease in BWF1 mice, is the major site of the development of T_{reg} cells.[6] Disruption of the architecture of the thymic medulla where development of T_{reg} cells occurs is known to impair that process.[28] To determine whether T_{reg} cells are properly

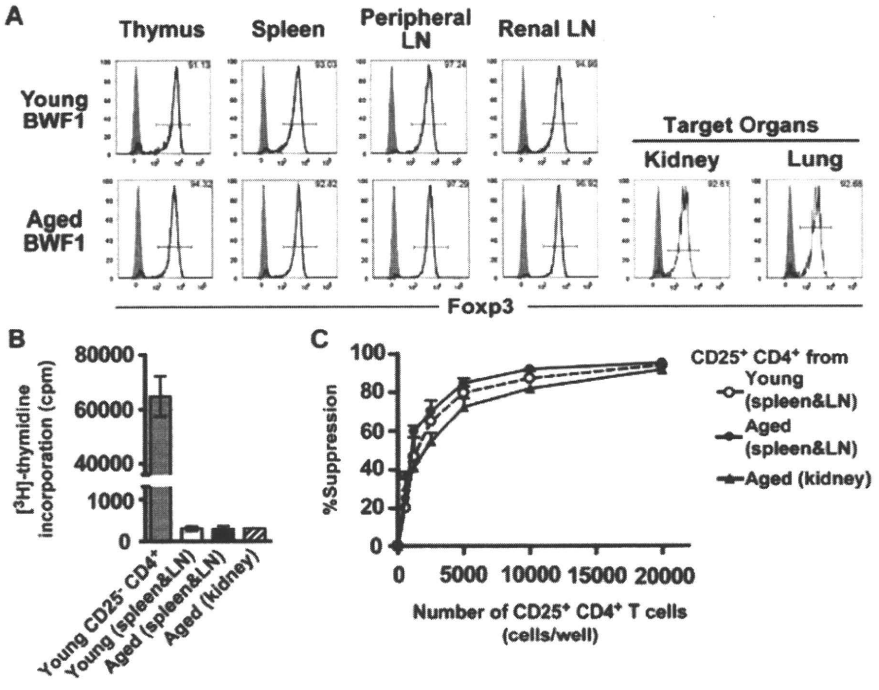

Figure 2. Suppressive activity of $CD25^+$ $CD4^+$ T cells. **A:** Representative profile of Foxp3 expression in $CD25^+$ $CD4^+$ T cells of young or aged BWF1 mice used for suppression assay ($n = 3$ for each group). Numbers in the histograms indicate the frequency of $Foxp3^+$ cells. Shaded histogram indicates isotype control. Note that $CD25^+$ $CD4^+$ T cells are highly enriched for $Foxp3^+$ T_{reg} cells. **B:** Proliferation of $CD25^+$ $CD4^+$ T cells isolated from the spleen and lymph nodes of young or aged BWF1 mice or from the target organs of aged BWF1 mice. Data are presented as mean ± SEM. **C:** *In vitro* suppressive activity of $CD25^+$ $CD4^+$ T cells isolated from the spleen and lymph nodes of young or aged BWF1 mice or from the kidney of aged BWF1 mice. Data were presented as mean + SEM. Differences between the three groups presented in the graph were not significant when analyzed by one-way analysis of variance with Bonferroni's multiple comparison test. A representative of three independent experiments that gave similar results is shown.

located within the thymus, we analyzed thymic sections by immunofluorescent staining. In BWF1 mice, thymic architecture is strongly affected by the disease,[4,29] but medullary localization of T_{reg} cells remained virtually unchanged even after the manifestation of severe nephritis (Figure 3, D and E). Localization of T_{reg} cells within the thymus is also confined to the medulla in BALB/c mice irrespective of their age (Supplemental Figure 1, A and B, see *http://ajp.amjpathol.org*).

Altered Distribution of T_{reg} Cells within the Secondary Lymphoid Organs of Aged BWF1 Mice

T_{reg} cells have to be located in the site of antigen presentation within the secondary lymphoid organs to make contacts with their target cells.[26,27] Because our analyses on the number, function, and site of the development of T_{reg} cells could not find any obvious defect, we examined the localization of T_{reg} cells within the secondary lymphoid organs of BWF1 mice. T_{reg} cells in aged BWF1 mice were located in the follicles and red pulp as well as periaortic lymphoid sheath in the spleen, whereas T_{reg} cells in young BWF1 mice were mostly located in the periaortic lymphoid sheath (Figure 3, F and G; Supplemental Figure 2, see *http://ajp.amjpathol.org*). Similar localization of T_{reg} cells were observed in the renal lymph node where T_{reg} cells were located in the follicles and medulla as well as paracortex in aged BWF1 mice, whereas the localization of T_{reg} cells in young BWF1 mice was relatively confined to paracortex (Figure 3, H and I; Supplemental Figure 2, see *http://ajp.amjpathol.org*). Such altered localization was not limited to T_{reg} cells, but was also seen in $Foxp3^-$ conventional T cells. In contrast, localization of T_{reg} cells in BALB/c mice was not altered with age and was similar to that of young BWF1 mice (Supplemental Figures 1, C–F, and 2, see *http://ajp.amjpathol.org*).

Changes in the Expression of Chemokine Receptors on T_{reg} Cells in Aged BWF1 Mice

Localization of T cells is tightly regulated by various chemokines and their receptors to achieve efficient induction of immune response or tolerance.[30] To elucidate the molecule(s) responsible for the altered localization of T_{reg} cells, we next analyzed the expression of chemokine receptors involved in the function of T_{reg} cells[31–34] by flow cytometry. Both $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells in the spleen showed decreased CCR7 expression (Figure 4C) and enhanced CXCR4 expression (Figure 4E) in aged BWF1 mice. On the other hand, the expression level of CCR4, CCR5, and CXCR3 did not show marked difference between young and aged BWF1 mice (Figure 4, A, B, and D), except that CXCR3 expression was slightly enhanced on both $Foxp3^+$ and $Foxp3^-$ cells of aged BWF1 mice (Figure 4, F and H). These changes in the expression of chemokine receptors on $CD4^+$ T cells were not observed in BALB/c mice (Figure 4, G and I). Expression pattern of chemokine receptors on $CD4^+$ T cells in the target organs and lymph nodes was similar to that of splenic $CD4^+$ T cells (data not shown). Aged BWF1 mice showed a 5 to 7 fold decrease in the expression of CCL19, CCL21, and CXCL12, ligands for CCR7 and CXCR4, in the lymphoid organs (Supplemental Figure 3, see *http://ajp.amjpathol.org*). On the other hand, expression of CXCL9 and CXCL10, ligands for CXCR3, were increased 2- to 3-fold and 8- to 28-fold, respectively, in

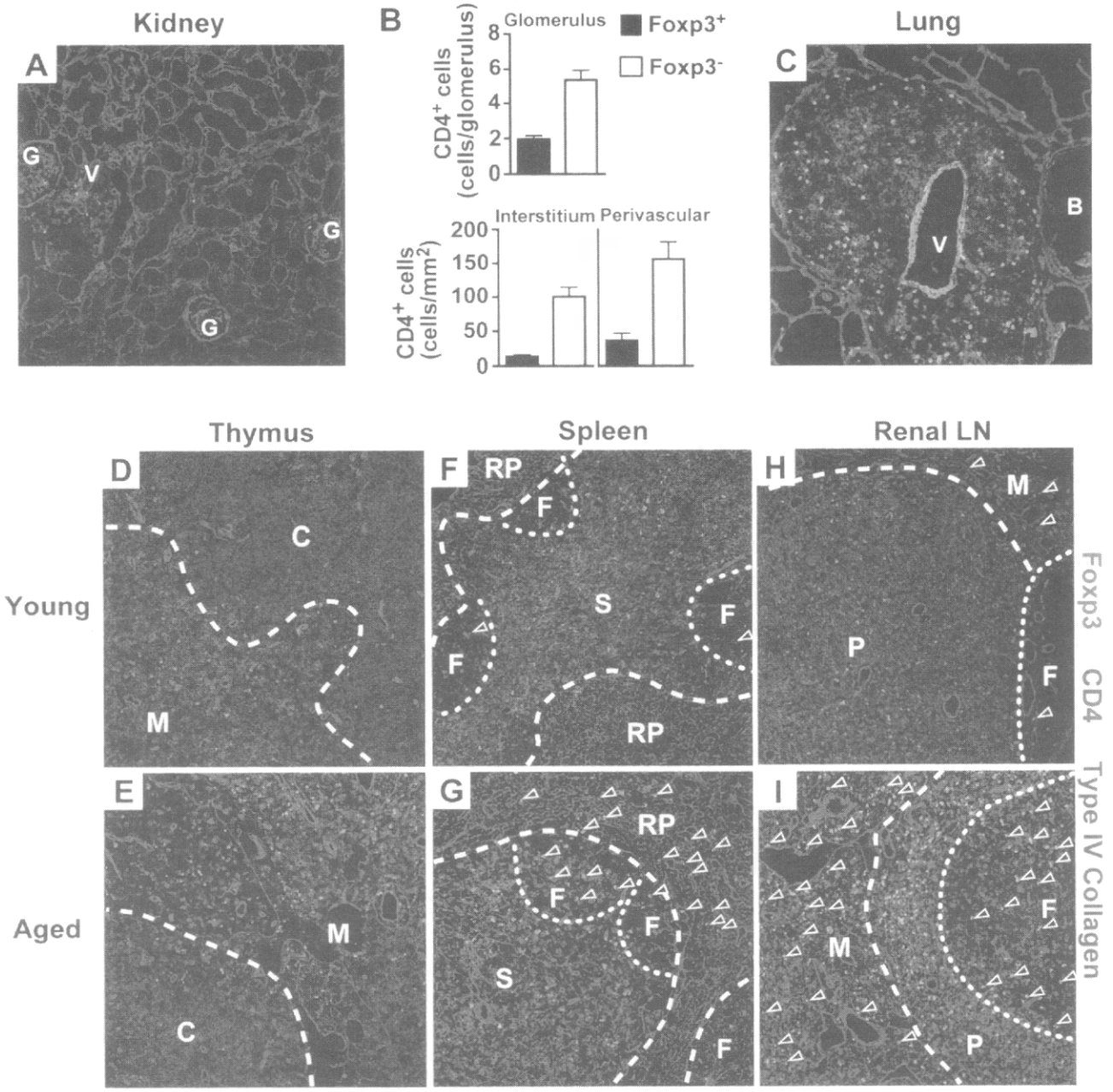

Figure 3. Altered localization of T_{reg} cells in aged BWF1 mice. **A–C:** Histological analysis of the kidney and lung of aged BWF1 mice (n = 4). **A** and **C:** Triple immunofluorescent staining of a 6-μm-thick cryosection of the kidney (**A**) and lung (**C**) of aged BWF1 mice with anti-Foxp3 (green), anti-CD4 (red), and anti-type IV collagen (blue). Green signal on the vascular endothelium and bronchus of the lung was also detected in isotype control (data not shown). Such nonspecific signal was not observed in $CD4^+$ cells. **B:** Summary of the number of $Foxp3^+$ (filled bar) and $Foxp3^-$ (open bar) $CD4^+$ T cells within renal compartments. Data were expressed as mean ± SEM. More than three fields were counted to calculate the mean value. **D–I:** Triple-immunofluorescent staining of 6-μm-thick cryosection of the thymus (**D**, **E**), spleen (**F**, **G**), and renal lymph node (**H**, **I**) of young (**D**, **F**, **H**) or aged (**E**, **G**, **I**) BWF1 mice with anti-Foxp3 (green), anti-CD4 (red), and anti-type IV collagen (blue). B, bronchus; C, cortex; F, follicle; G, glomerulus; M, medulla; RP, red pulp; P, paracortex; S, periaortic lymphoid sheath; V, blood vessel. **Arrowheads** in **D–I** indicate $Foxp3^+$ $CD4^+$ T cells located out of paracortex or periaortic lymphoid sheath. Representatives of the independent examination of four young and aged BWF1 mice are shown. Original magnifications, ×100.

the lymphoid organs and target organs, respectively (Supplemental Figure 3, see *http://ajp.amjpathol.org*).

Activated Phenotype of Both $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T Cells in Aged BWF1 Mice

Altered localization of T_{reg} cells in aged BWF1 mice per se does not explain the cause of their failure to control the autoimmunity. We found that T_{reg} cells of aged BWF1 mice showed decreased expression of CD25 and CD62L (Figure 5, A, B, and I), in contrast to the enhanced or unaltered expression of activation markers CD44, CD69, and CD103 (Figure 5, C–E, and I). Various co-stimulatory molecules up-regulated on activation were reported to affect the function and/or number of T_{reg} cells,[35–37] therefore, we analyzed the expression of co-stimulatory molecules of

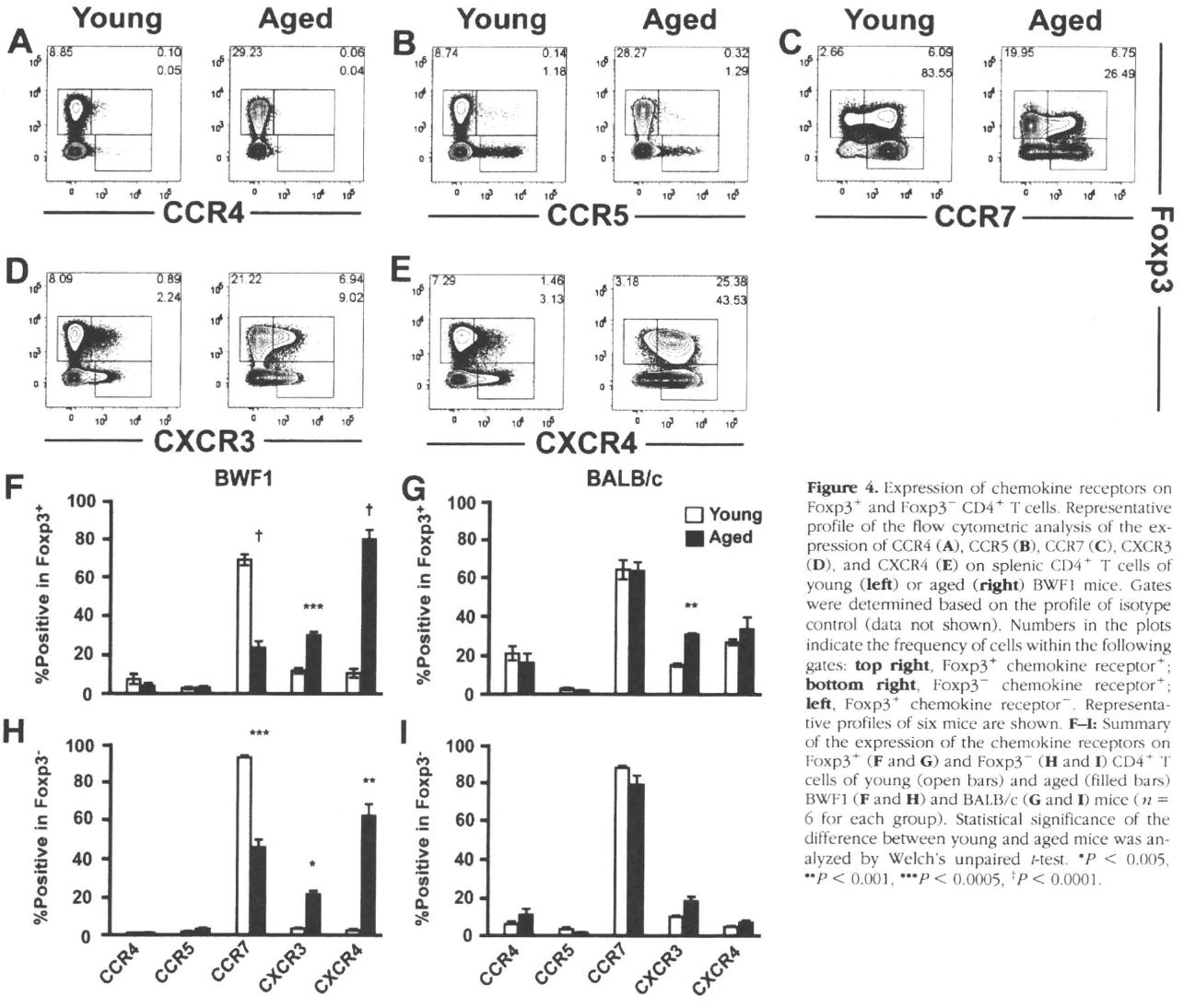

Figure 4. Expression of chemokine receptors on Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells. Representative profile of the flow cytometric analysis of the expression of CCR4 (**A**), CCR5 (**B**), CCR7 (**C**), CXCR3 (**D**), and CXCR4 (**E**) on splenic CD4$^+$ T cells of young (**left**) or aged (**right**) BWF1 mice. Gates were determined based on the profile of isotype control (data not shown). Numbers in the plots indicate the frequency of cells within the following gates: **top right**, Foxp3$^+$ chemokine receptor$^+$; **bottom right**, Foxp3$^-$ chemokine receptor$^+$; **left**, Foxp3$^+$ chemokine receptor$^-$. Representative profiles of six mice are shown. **F–I:** Summary of the expression of the chemokine receptors on Foxp3$^+$ (**F** and **G**) and Foxp3$^-$ (**H** and **I**) CD4$^+$ T cells of young (open bars) and aged (filled bars) BWF1 (**F** and **H**) and BALB/c (**G** and **I**) mice (n = 6 for each group). Statistical significance of the difference between young and aged mice was analyzed by Welch's unpaired t-test. $^*P < 0.005$, $^{**}P < 0.001$, $^{***}P < 0.0005$, $^{\dagger}P < 0.0001$.

T_{reg} cells. Associated with their activated phenotype, costimulatory molecules OX40, 4-1BB, and ICOS were expressed on CD4$^+$ T cells in aged BWF1 mice at higher level than young BWF1 (Figure 5, F–I). Among them, expression of OX40 and ICOS was enhanced on both Foxp3$^+$ and Foxp3$^-$ T cells, whereas expression of 4-1BB was enhanced only on Foxp3$^+$ T_{reg} cells (Figure 5, I and K). Age-dependent alteration of surface phenotype in BALB/c mice was limited to slight changes in CD44 and CD62L (Figure 5, J and L).

Inability of T_{reg} Cells of BWF1 Mice to Suppress in Vitro *IgG Antibody Production*

Lastly, we assessed the impact of T_{reg} cells on the antibody production by B cells. Sekigawa and colleagues[24] demonstrated that CD4$^+$ T cells of aged BWF1 mice induced IgG antibody production of splenic B cells on stimulation with concanavalin A and lipopolysaccharide *in vitro*. We used this method with several modifications and found that CD25$^-$ CD4$^+$ T cells of aged, but not young, BWF1 mice induced IgG antibody production by B cells even in the absence of the stimuli (Figure 6 and data not shown). Because antibody production by B cells was totally dependent on the presence of CD4$^+$ T cells in this assay, we assumed that T_{reg} cells would suppress the antibody production by interfering with CD4 help. CD25$^+$ CD4$^+$ T cells, however, did not affect the amount of IgG antibody produced by B cells (Figure 6), demonstrating that T_{reg} cells of both young and aged BWF1 mice are unable to suppress IgG antibody production induced by CD4$^+$ T cells of aged BWF1 mice.

Discussion

Foxp3$^+$ CD4$^+$ T_{reg} cells play a pivotal role in the maintenance of dominant self tolerance, and lack of functional T_{reg} cells is associated with various autoimmune diseases. In contrast, our present study in a murine model of SLE revealed a substantially expanded pool size of T_{reg} cells with a phenotype suggesting their highly activated state, and their inability to suppress antibody production *in vitro*.

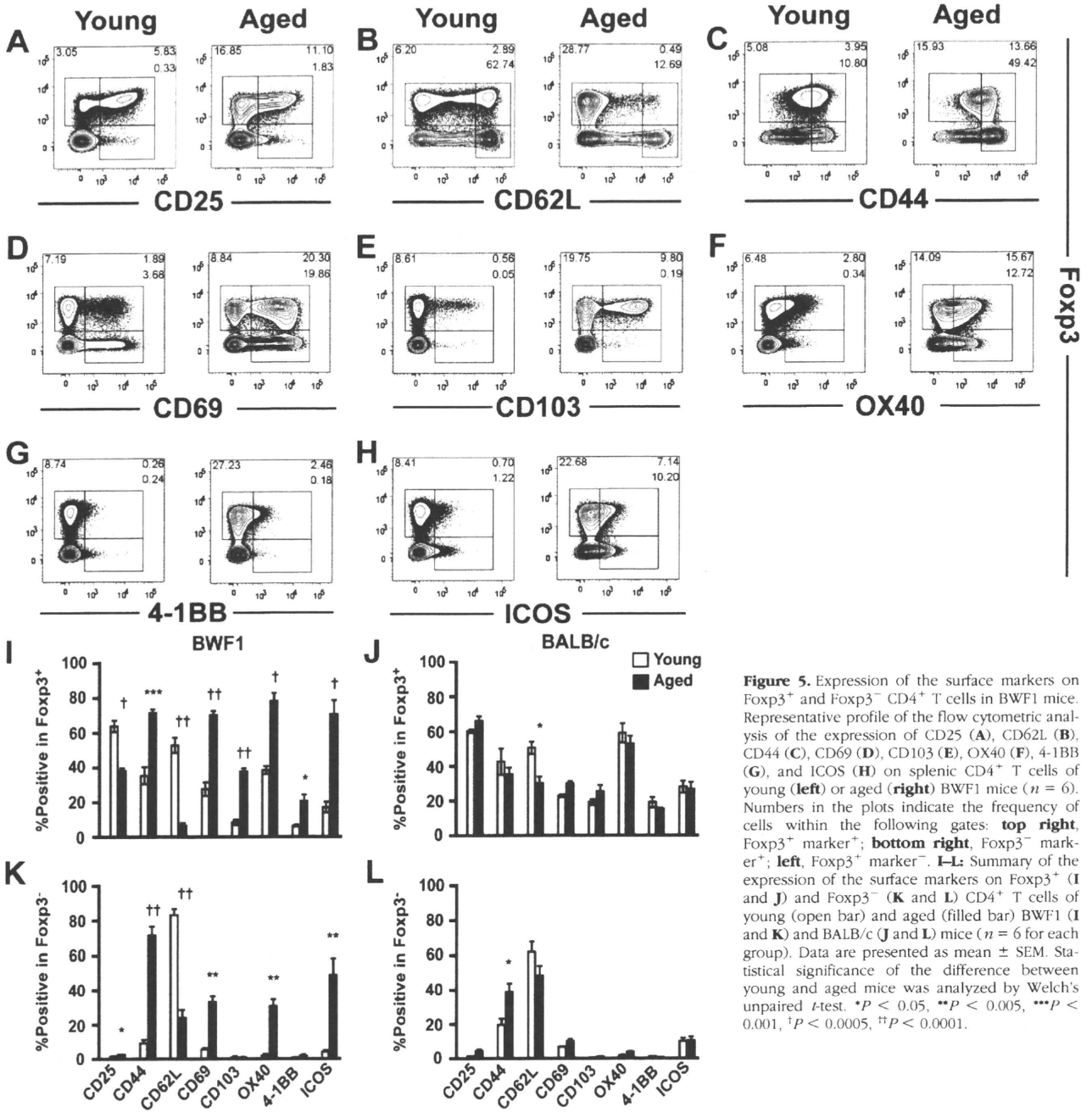

Figure 5. Expression of the surface markers on $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells in BWF1 mice. Representative profile of the flow cytometric analysis of the expression of CD25 (**A**), CD62L (**B**), CD44 (**C**), CD69 (**D**), CD103 (**E**), OX40 (**F**), 4-1BB (**G**), and ICOS (**H**) on splenic $CD4^+$ T cells of young (**left**) or aged (**right**) BWF1 mice ($n = 6$). Numbers in the plots indicate the frequency of cells within the following gates: **top right**, $Foxp3^+$ $marker^+$; **bottom right**, $Foxp3^-$ $marker^+$; **left**, $Foxp3^+$ $marker^-$. **I–L:** Summary of the expression of the surface markers on $Foxp3^+$ (**I** and **J**) and $Foxp3^-$ (**K** and **L**) $CD4^+$ T cells of young (open bar) and aged (filled bar) BWF1 (**I** and **K**) and BALB/c (**J** and **L**) mice ($n = 6$ for each group). Data are presented as mean ± SEM. Statistical significance of the difference between young and aged mice was analyzed by Welch's unpaired *t*-test. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{\dagger}P < 0.0005$, $^{\dagger\dagger}P < 0.0001$.

We could not detect any obvious defect in the suppressive activity of T_{reg} cells in BWF1 mice. In addition, localization of both T_{reg} cells and $Foxp3^-$ conventional $CD4^+$ T cells within the lymphoid organs was altered, but they showed concomitant migratory behavior. These data collectively suggest that T_{reg} cells in BWF1 mice had little defect in their function, and the failure of T_{reg} cells to control the disease might be predominantly caused by the extrinsic factors, such as cytokine milieu and costimulatory signals provided by antigen-presenting cells (APCs). On the other hand, it is reported that treatment of BWF1 mice with the T_{reg} cell-inducing molecules such as all-*trans*-retinoic acid or tolerogenic peptides delays or prevents the onset of murine lupus.[18–20,38] One possible explanation for the failure of T_{reg} cells to control the disease is that presence of T_{reg} cells capable of controlling the disease at an earlier stage is critical, as suggested by the previous reports in which induction of T_{reg} cells in BWF1 mice was conducted well before the onset of the disease. Another possibility is the antigen specificity of T_{reg} cells. La Cava and colleagues[19] showed that induction of T_{reg} cells specific for the peptide derived from anti-DNA antibody were associated with the therapeutic effect of this peptide in BWF1 mice. This report raises the possibility that endogenous T_{reg} cells in prediseased BWF1 mice lack population(s) with such antigen specificity, and expansion of the pool size of T_{reg} cells in aged BWF1 mice with severe nephritis does not

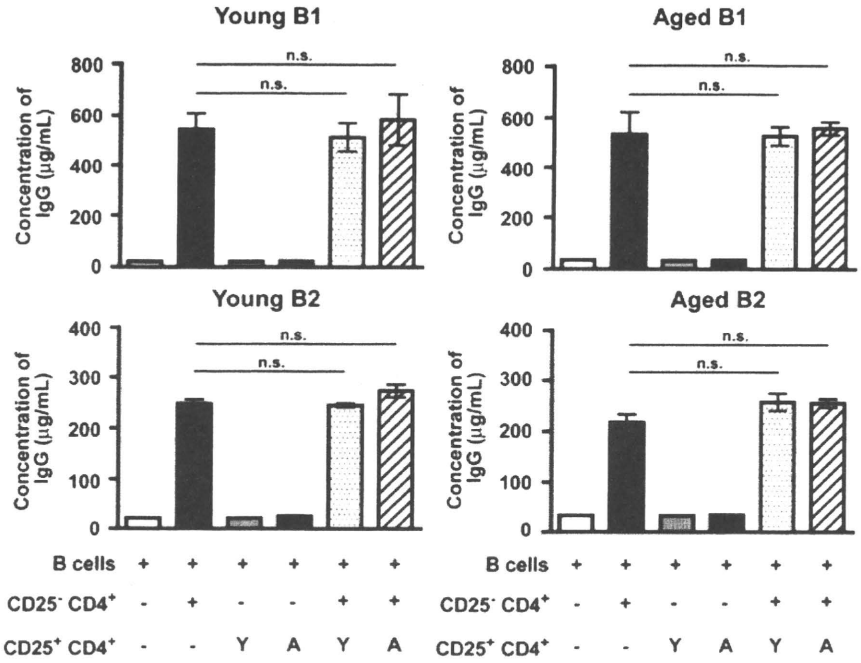

Figure 6. Inability of T_{reg} cells of BWF1 mice to suppress *in vitro* antibody production induced by $CD25^-$ $CD4^+$ T cells of aged BWF1 mice. Concentration of IgG antibody in the culture supernatant was measured by enzyme-linked immunosorbent assay after co-culture of T cells and B cells in the following combinations for 5 days. B cells alone, (white column); B cells + $CD25^-$ $CD4^+$ T cells (black column); B cells + $CD25^+$ $CD4^+$ T cells of young BWF1 (light gray column); B cells + $CD25^+$ $CD4^+$ T cells of aged BWF1 (dark gray column); B cells + $CD25^-$ $CD4^+$ T cells + $CD25^+$ $CD4^+$ T cells of young BWF1 (dotted column); B cells + $CD25^-$ $CD4^+$ T cells + $CD25^+$ $CD4^+$ T cells of aged BWF1 (striped column). $CD25^-$ $CD4^+$ T cells of aged BWF1 mice were used for all combinations. B-cell subsets used for each combination were indicated above each panel. Data are presented as mean ± SEM. n.s., not significant by one-way analysis of variance with Bonferroni's multiple comparison test. Representative of three independent experiments is shown.

compensate for that repertoire. It is therefore feasible that accumulation of T_{reg} cells is too late to control the pathogenic autoimmune response in aged BWF1 mice, or that antigen specificity of T_{reg} cells in aged BWF1 mice differ from those in young BWF1 mice. However, there are other possible mechanisms for the inability of T_{reg} cells to control the pathogenic autoimmune response in aged BWF1 mice as described below.

There are several reports suggesting a possible effect of T_{reg} cells on T-dependent B-cell responses.[19,39–41] It was, therefore, surprising that T_{reg} cells of BWF1 mice could not suppress the *in vitro* antibody production induced by $CD25^-$ $CD4^+$ T cells despite their intact suppressive activity against the proliferation of T cells *in vitro*. Possible explanations for our result are as follows: first, loss of the sensitivity of $CD25^-$ $CD4^+$ T cells of aged BWF1 mice to T_{reg} cell-mediated suppression; second, reversal of T_{reg} cell-mediated suppression by signaling through co-stimulatory molecules. OX40, 4-1BB, and ICOS have been implicated in the pathogenesis of lupus.[42–44] OX40 and 4-1BB magnify the T-cell response through induction of the proliferation of conventional T cells and inhibition of T_{reg} cell-mediated immune suppression.[37,45] The ICOS-mediated signal is essential for the induction of follicular helper T cells, thus it functions as an enhancer of B-cell response.[46] On the contrary, these molecules as well as ICOS also facilitate the expansion of T_{reg} cells.[36,45,47] B cells of aged BWF1 mice, however, did not show significant expression of ligands for these co-stimulatory molecules (data not shown). This observation implies that reversal of the suppression, if any, might take place through the other pathway(s). Also, $CD25^-$ $CD4^+$ T cells of aged, but not young, BWF1 mice contain $CXCR5^+$ $ICOS^+$ follicular helper T cells whose function may be resistant to T_{reg} cell-mediated suppression. Further studies with regard to the impact of T_{reg} cells on humoral immune response as well as the interaction between T_{reg} cells and their target cells will be required to clarify their role in antibody-mediated autoimmune diseases such as SLE.

Concomitant migratory behavior of T_{reg} cells and conventional T cells was shown to be crucial for the immunoregulatory function of T_{reg} cells.[26,31,32] Chemokines and their receptors, as well as the activation markers CD44, CD62L, CD69, and CD103, are the possible regulators of the migration of T cells. Our present data demonstrating similar localization of T_{reg} cells and conventional T cells with the comparable expression of chemokine receptors and activation markers between these cells suggest that regulation of the migratory behavior of these cells were not impaired; however, BWF1 mice still develop the fatal autoimmune response. This idea, together with our notion of intact suppressive activity, further suggests that failure of T_{reg} cells to control the disease is because of the other factor(s) residing in the microenvironment.

Collectively, we demonstrated that aged BWF1 mice developing lupus nephritis had increased $Foxp3^+$ $CD4^+$ T_{reg} cells with highly activated phenotype and altered localization, but with intact suppressive activity. Our present results may provide a clue to understanding the nature of T_{reg} cells in the lupus and also help to unveil the mechanisms of the failure of T_{reg} cells to control autoimmune responses. Further studies directed at these points would facilitate the development of novel strategies for the treatment of SLE.

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Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis: RRR (remission induction by Remicade in RA) study

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ABSTRACT

Background Tumour necrosis factor (TNF) inhibitors enable tight control of disease activity in patients with rheumatoid arthritis (RA). Discontinuation of TNF inhibitors after acquisition of low disease activity (LDA) is important for safety and economic reasons.

Objective To determine whether infliximab might be discontinued after achievement of LDA in patients with RA and to evaluate progression of articular destruction during the discontinuation.

Methods 114 patients with RA who had received infliximab treatment, and whose Disease Activity Score, including a 28-joint count (DAS28) was <3.2 (LDA) for 24 weeks, were studied.

Results The mean disease duration of the 114 patients was 5.9 years, mean DAS28 5.5 and mean modified total Sharp score (mTSS) 63.3. After maintaining LDA for >24 weeks by infliximab treatment, the drug was discontinued and DAS28 in 102 patients was evaluated at year 1. Fifty-six patients (55%) continued to have DAS28<3.2 and 43% reached DAS<2.6 at 1 year after discontinuing infliximab. For 46 patients remission induction by Remicade in RA (RRR) failed: disease in 29 patients flared within 1 year and DAS28 was >3.2 at year 1 in 17 patients. Yearly progression of mTSS (ΔTSS) remained <0.5 in 67% and 44% of the RRR-achieved and RRR-failed groups, respectively. The estimated ΔmTSS was 0.3 and 1.6 and Health Assessment Questionnaire-Disability Index was 0.174 and 0.614 in the RRR-achieved and RRR-failed groups, respectively, 1 year after the discontinuation.

Conclusion After attaining LDA by infliximab, 56 (55%) of the 102 patients with RA were able to discontinue infliximab for >1 year without progression of radiological articular destruction.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that causes significant morbidity and mortality. The combined use of biological agents targeting tumour necrosis factor (TNF) and methotrexate (MTX) has produced significant improvements in clinical, radiographic and functional outcomes that were not previously seen and has revolutionised the treatment goal of RA to clinical remission, structural remission and functional remission.[1–5] The next goal should be remission without the use of biological agents and subsequent drug-free remission. Although global evidence of the efficacy and safety of TNF inhibitors such as infliximab has accumulated, including the ATTRACT study, ASPIRE study, our RECONFIRM studies and many others,[5–10] there is no well-established firm evidence for remission free from the use of biological agents.

The initial report of the potential for remission without the use of biological agents in patients with RA was reported by a British group (TNF20 study).[11] The combination of infliximab and MTX in patients with early RA who had fewer than 12 months of symptoms provided tight control of the disease activity and a significant reduction in MRI evidence of synovitis and erosions at 1 year. At 2 years, functional and quality of life benefits were sustained, despite withdrawal of infliximab treatment. On the other hand, the Behandelstrategieën (BeSt) study was conducted to observe clinical and radiological outcomes of patients with early RA treated with initial infliximab and MTX who discontinued infliximab after achieving a sustained Disease Activity Score (DAS) ≤2.4. Five years after receiving infliximab and MTX as initial treatment for RA, 58% of 120 patients discontinued infliximab because of a continuous DAS ≤2.4 and 19% of patients have stopped all antirheumatic drugs and remain in clinical remission, with minimal joint damage progression. These findings indicate that treatment using infliximab and MTX, guided by DAS, is an effective and tight control to maintain low disease activity (LDA) and may alter the course of early RA.[12–16]

Discontinuation of TNF inhibitors after acquisition of LDA is important for reasons of safety and economy. For instance, the problem of the incidence of haematological malignancy owing to the long-term use of TNF inhibitors remains unresolved. In Japan a large majority of patients have to pay 30% of their medical costs and all wish to know for how long biological agents must be continued, but we have no answer. We successfully discontinued infliximab after attaining DAS-guided remission for >24 weeks,[10] but evidence based on multicentre studies is needed. Reports published to date on this topic are confined to those from the BeSt study and TNF20 study involving only patients at an early stage of RA.[11–16]

Thus, this multicentre study was undertaken to seek the possibility of discontinuing infliximab after attaining DAS-guided remission and maintaining LDA without infliximab, in patients with RA, including patients with long-established disease, and to evaluate progression of articular destruction and functional disabilities during the discontinuation.

PATIENTS AND METHODS

Patients

Data and information on patients with RA fulfilling the diagnostic criteria of the American College of Rheumatology were collected from 26 centres of remission induction by Remicade in RA (RRR) investigator groups in Japan.[17] Disease activity of individual patients was assessed by Disease Activity Score, including a 28-joint count (DAS28)-erythrocyte sedimentation rate (ESR) or DAS28-C reactive protein (CRP) that was calculated according to the authorised formula (http://www.das-score.nl/, accessed 15 February 2010).[18] Since none of the patients, except for one, achieved LDA measured by DAS28 despite MTX or a combination of MTX and other disease-modifying antirheumatic drugs for at least 3 months, infliximab treatment (3 mg kg, every 8 weeks) was administered in the investigators' institutions, according to the treatment guideline proposed by the Japan College of Rheumatology.[17] Joint damage was assessed by the van der Heijde-modified total Sharp score (mTSS)[19] and for 102 patients, x-ray images of the hands and feet at baseline, RRR-study entry and 1 year after the study were available; these were evaluable for 49 patients owing to loss of the radiographs and/or low quality of the x-ray images.[20 21] Two blinded expert readers independently scored articular damage and progression according to the mTSS scoring method. The difference between the two readers' scores for each patient's radiographs was <1% of the maximum mTSS score—that is, 448.[9 20 21] To confirm that the x-ray results of the 49 patients represented the outcomes of the whole group, we compared multiple background characteristics and changes of each characteristic from baseline to RRR-study entry between 49 patients with evaluable x-ray images and 53 patients without them and no significant difference was seen between the two groups.

After patients had achieved DAS28 (ESR)<3.2 (LDA) for >24 weeks, informed consent to discontinue infliximab was obtained from 126 patients. Other criteria were that patients were controlled with <5 mg/day of oral prednisolone (PSL) and were >18 years old. Concomitant use of MTX was started in all patients, and the dose of MTX was determined by each attending doctor. Twelve patients dropped out at the screening period, and 114 patients were enrolled in the study and discontinued infliximab (figure 1). The demographic indicators and baseline disease characteristics of the 114 patients enrolled are summarised in table 1.

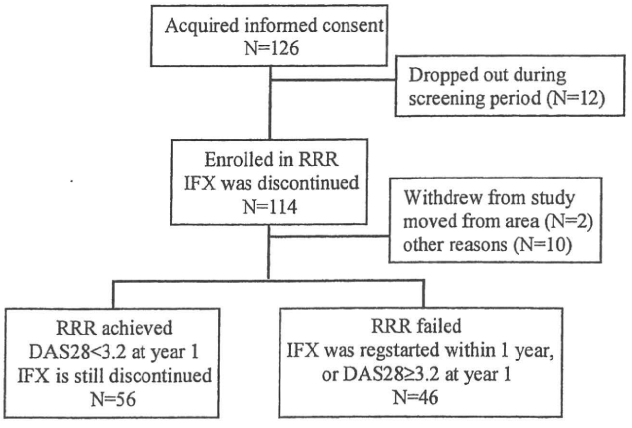

Figure 1 Study design and profile. DAS28, Disease Activity Score, including a 28-joint count; IFX, infliximab; RRR, remission induction by Remicade in rheumatoid arthritis.

Table 1 Demographic indicators and baseline disease characteristics

	Enrolled patients (N=114)	RRR-achieved (N=56)	RRR-failed (N=46)	p (probability >χ^2)
Women	87 (76%)	42 (75%)	38 (83%)	0.4691
Age (years)	51.4 (20.0–73.0)	49.5±12.6	56.1±12.2	*0.0053*
Disease duration (years)	5.9 (0.1–38.0)	4.8±5.9	7.8±7.7	*0.0238*
Tender joint count (0–28)	8.2±6.7	8.6±7.0	7.5±5.8	0.5798
Swollen joint count (0–28)	9.0±7.2	10.1±7.7	7.6±5.8	0.1674
PaGA (0–100 mm, VAS)	50.0±23.0	50.0±24.2	49.3±23.1	0.9520
CRP (mg/dl)	2.5±3.0	2.6±2.6	2.7±3.7	0.5531
ESR (mm/h)	46.2±26.9	43.1±24.2	54.1±30.1	0.1555
DAS28 (ESR) score	5.5±1.2	5.5±1.4	5.6±1.1	0.9112
DAS28 (CRP) score	4.9±1.2	5.1±1.3	4.8±1.3	0.5486
HAQ-DI	1.0±0.7	0.9±0.6	1.2±0.7	0.1112
mTSS*	63.3 (1.0–314.0)	46.9±46.5	97.2±86.9	*0.0207*
RF (U/ml)	201.9±496.5 (68.5%)	225.7±583.3	197.9±427.8	0.5190
MTX (mg/week)	7.7±2.3	7.9±1.9	7.8±2.8	0.3232
PSL (mg/day)	2.5±3.4 (45.6%)	2.4±3.5	2.8±3.5	0.5223

Data are number of patients (%) for categorical data and the means for continuous data. Statistical difference was assessed by non-parametric Wilcoxon t test and p (probability >χ^2) values are shown. Values in italic indicate a significant difference ($p<0.05$).
*Data supplied for 33 patients who achieved RRR and 16 patients for whom RRR failed.
CRP, C-reactive protein; DAS28, Disease Activity Score, including a 28-joint count; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; mTSS, modified total Sharp score; MTX, methotrexate; PaGA, patient global assessment of disease activity; PSL, prednisolone; RF, rheumatoid factor; RRR, remission induction by Remicade in rheumatoid arthritis; VAS, visual analogue scale.

Procedures

Study protocol was a simple observation after discontinuation of infliximab. The follow-up observation was monitored by symptoms, signs and DAS28 (ESR) every 4–13 weeks for 2 years. The dose of concomitant MTX was basically consistent, but tapering of non-steroidal anti-inflammatory drugs and glucocorticoid was allowed during the study period. The primary end points were that after discontinuing infliximab, DAS28 remains <3.2 (LDA) for 1 year and (B) yearly progression of mTSS remains <0.5 (structural remission) for 1 year. Secondary end points were DAS28 remains <2.6 (clinical remission) for 1 year, DAS28 remains <3.2 for 2 years, yearly progression of mTSS remains <0.5 for 2 years and no rescue with infliximab for 1 or 2 years is needed, after discontinuing infliximab. When a flare-up occurred in patients after the discontinuation, restart of infliximab was allowed and patients were categorised into the 'RRR-failed' group. For the restart of infliximab, the same dose (3 mg/kg) and the same premedication as used before the study entry were used.

Statistical analysis

Baseline characteristics of patients are summarised in table 1 using the mean values for continuous variables. All multivariate analyses were conducted using the variables gender, age, duration of disease, DAS28 (ESR) score, DAS28 (CRP) score, tender joint count (0–28), swollen joint count (0–28), patient global assessment of disease activity (PaGA, 0–100 mm, visual analogue scale), ESR, CRP, Health Assessment Questionnaire-Disability Index (HAQ-DI), rheumatoid factor (RF), MTX dose and PSL dose at baseline. Spearman correlation analyses were performed to evaluate the

association between multivariables at RRR-study entry and DAS28 at the primary end point (last observation carried forward) of 102 patients. Logistic regression analysis was carried out to estimate DAS28 at the primary end point as dependent variables (probability) by DAS28 at RRR entry as independent variables. A receiver operating characteristic (ROC) curve was developed based on the logistic analysis and the significant cut-off point was determined from the curve. For categorical response parameters, group comparisons were made using a non-parametric Wilcoxon t test. Statistical analyses were performed using JMP software version 7 (SAS Institute, Cary, North Carolina, USA). All reported p values are two sided and p values <0.05 were considered significant.

RESULTS

Study end points

The demographic indicators and baseline characteristics of the 114 patients enrolled were as follows: mean age 51.4 years, mean disease duration 5.9 years and mean mTSS 63.3, indicating that the population included patients with long-established disease, and the mean DAS28 (ESR) score was 5.5, implying that most patients had highly active disease (table 1). Figure 1 shows the study profile. After maintaining DAS28<3.2 (LDA) for >24 weeks by infliximab treatment, infliximab was discontinued in 114 patients. Twelve patients withdrew because they moved from area (n=2) and for other reasons (n=10), and thus DAS28 could be evaluated in 102 patients at year 1.

Of the 102 patients, 56 patients achieved the primary end point having a DAS28<3.2 and remaining without infliximab for 1 year after the discontinuation (figure 2A). Thus, 55% of the enrolled patients met the primary end point that LDA was maintained for 1 year after discontinuing infliximab. Furthermore, 44 patients (43%) reached DAS<2.6 after the discontinuation. On the other hand, 29 patients flared within 1 year (mean duration was 6.4 months) after the discontinuation and in 17 patients DAS28 was >3.2 at year 1 and thus RRR failed for 46 patients (45%) at year 1. Re-treatment with infliximab in 32 patients was effective and the majority of patients reached DAS28<3.2 within 24 weeks (figure 2B). Minimal adverse reactions at infusion of the agent were seen in five patients only at the first or second infusion.

To clarify the background factors related to the RRR-achievement, multiple clinical parameters at baseline were compared between patients for whom RRR was achieved and those for whom it failed. Patients for whom RRR was achieved were younger (49.5 vs 56.1), their disease duration was shorter (4.8 vs 7.8) and mTSS was lower (46.9 vs 97.2) than for those for whom RRR failed. Among 56 patients who achieved RRR, 10 patients had early RA (disease duration <1.0 year) and eight long-established disease (>10 years). Of 46 patients for whom RRR failed, eight had early RA and 12 established disease. These results imply that infliximab can be discontinued in patients with long-established RA. In contrast, no significant difference was seen in gender, DAS including DAS28, tender or swollen joint count, ESR and CRP, HAQ-DI, RF and the dose of MTX and PSL. Since these factors interact with one another, we analysed the relationship between RRR-achievement and a series of clinical parameters at baseline using multivariate analysis after adjusting for confounding variables. No significant relations

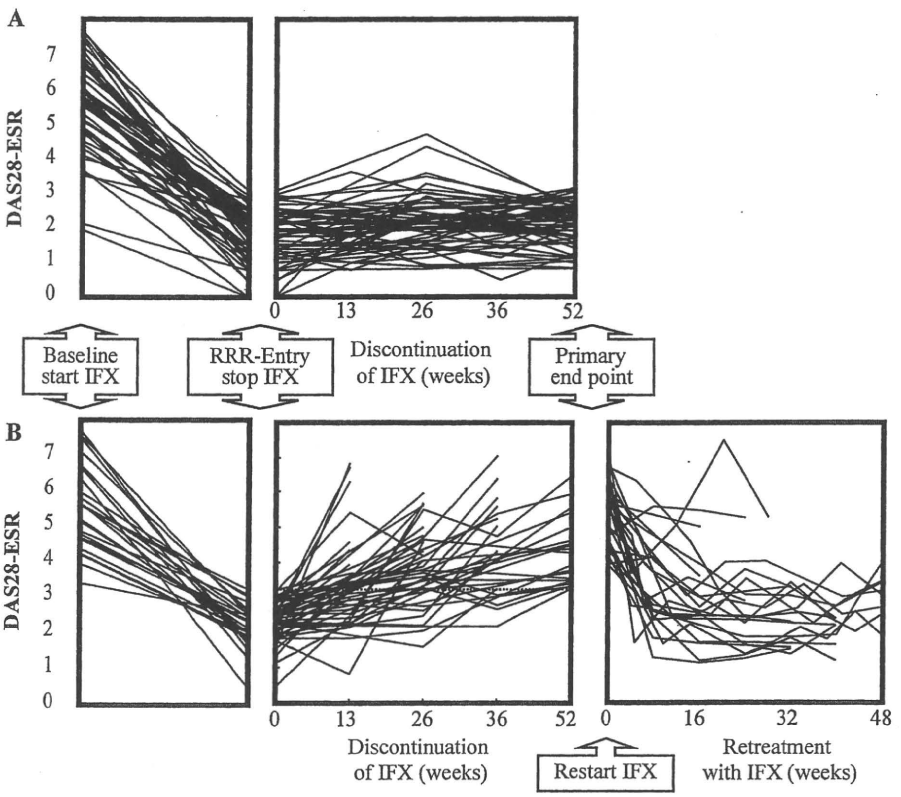

Figure 2 Changes of Disease Activity Score, including a 28-joint count (DAS28) in patients with remission induction by Remicade in rheumatoid arthritis-achieved (RRR-achieved) and patients for whom RRR failed (RRR-failed). (A) Changes of Disease Activity Score, including a 28-joint count (DAS28) at baseline when infliximab (IFX) was administered, at RRR-study entry when infliximab was discontinued and at the primary end point at week 52 after discontinuing IFX in 56 patients who were still satisfied with DAS28 (erythrocyte sedimentation rate (ESR)) <3.2 at week 52, RRR-achieved'. (B) Changes of DAS28 at baseline, at RRR entry and the end point in 46 patients whose disease activity flared after the discontinuation of IFX or DAS28 >3.2 at week 52, 'RRR-failed'. The lower right panel shows changes of DAS28 after the restarting IFX in 32 patients for whom RRR failed.

between RRR-achievement and age, gender, DAS28 (ESR) score, PaGA and CRP were found, whereas a significant correlation was found with disease duration (p=0.0019) and serum levels of RF (p=0.0128) in RRR-achievement.

To determine the correlation of DAS28 at the primary end point with clinical parameters at RRR-study entry, univariate analysis of multiple variables was carried out. No significant correlations between DAS28 (ESR) at the primary end point and a series of clinical parameters were found, whereas DAS28 (ESR) and DAS28 (CRP) at RRR-entry were significantly correlated with DAS28 (ESR) at the primary end point. Subsequently, logistic regression analysis to estimate the probability of DAS28<3.2 at the primary end point as dependent variable by DAS28 at RRR-entry as independent variable was assessed. A significant logistic regression curve was drawn between the dependent and independent variables (p=0.0005) (figure 3A). Thus, DAS28 at RRR-study entry had the most marked correlation with the maintenance of LDA for 1 year after the discontinuation. By reciprocal statistics, DAS28 at RRR-study entry was estimated as 2.22, to attain DAS28<3.2 at the end point in 50% of the 102 patients (figure 3A). Furthermore, 71.4% of patients whose DAS28 at study entry was <2.225, a cut-off point calculated from the ROC curve, continued to have DAS28<3.2 for 1 year, whereas only 32.6% of patients whose DAS28 at RRR-entry was 2.225–3.2 continued to have DAS28<3.2 (figure 3B), indicating that 'deep remission' was required to maintain lower disease activity for 1 year after discontinuation of infliximab.

Structural and functional changes

From the 102 patients enrolled in the study, 49 patients were selected in whom both hand and feet x-ray data were available and evaluable; experts examined the structural damage before and after the infliximab treatment. When the baseline characteristics of the 49 patients in the study were compared with the rest of the patients in the study with insufficient x-ray data (n=53), no significant difference was seen. Next, the baseline characteristics of the 33 patients who achieved RRR and 16 patients for whom RRR failed were compared. As described in table 1, disease duration was shorter and mTSS was lower in patients who achieved RRR than in patients for whom RRR failed, but yearly progression of mTSS (ΔmTSS) was comparable between two groups (table 2). ΔmTSS at RRR entry was also comparable between two groups. However, means (0.3 vs 1.6) and medians (0.0 vs 1.5) of ΔmTSS were lower in the RRR-achieved group than in the RRR-failed group and more patients in the RRR-achieved group (67%) achieved ΔmTSS<0.5, radiographic remission, than patients in the RRR-failed group (44%). Thus, another primary end point for structural remission was achieved for 1 year after the discontinuation. Furthermore, HAQ-DI at baseline and RRR entry was comparable between patients for whom RRR was achieved and those for whom it failed, whereas HAQ-DI at the primary end point in patients who achieved RRR was significantly lower than that in patients for whom RRR failed (0.174 vs 0.614) (figure 4).

DISCUSSION

This multicentre study was undertaken to determine the possibility of discontinuing infliximab treatment in patients with RA after acquiring DAS-guided LDA, including those with long-established disease. Among 102 patients who could be evaluated at year 1, 56 patients (55%) satisfied the primary end point by maintaining DAS28<3.2 (LDA) and 44 patients (43%) reached DAS<2.6 (remission), remaining without infliximab at year 1 after the discontinuation. Of the 102 patients, 83 (81.4%) were in clinical remission at study entry and after discontinuing infliximab, 39/83 patients (47%) remained in remission and 10/83 patients (12%) progressed to LDA at the primary end point.

These data are similar to those of the BeSt study. However, the greatest difference between the patient populations enrolled in the two studies is mean disease duration—0.4 years in the BeSt study versus 5.9 years in our RRR study.[12–16] Joint destruction also differed between the two studies—mean mTSS 7.0 in the BeSt study versus 63.3 in our RRR study—suggesting that discontinuation of infliximab after reaching LDA is possible in patients with early RA and also in patients with long-established disease.[13–15] On the other hand, among multiple clinical parameters at baseline, disease duration was statistically related to RRR-achievement by multivariate analysis and disease duration was shorter (4.8 vs 7.8) and mTSS was lower (46.9 vs 97.2) in patients who achieved

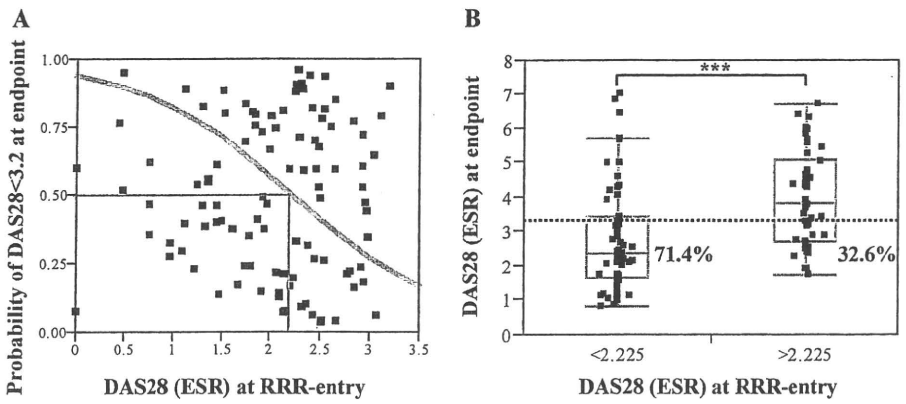

Figure 3 Logistic analysis of probability of Disease Activity Score, including a 28-joint count (DAS28) was <3.2 at primary end point by DAS28 at remission induction by Remicade in rheumatoid arthritis entry (RRR entry). (A) Logistic regression analysis to estimate DAS28 at primary end point as dependent variables by DAS28 at RRR entry as independent variables. The y-axis shows the probability of DAS28 <3.2 at the primary end point after the 52 weeks discontinuation of infliximab and a scatter diagram of an individual patient and logistic regression curve (solid line) are shown. To attain DAS28 <3.2 at the end point in 50% of the 102 patients, DAS28 at RRR study entry was estimated by reciprocal statistics. (B) From the receiver operating characteristic curve based on the logistic regression analysis above, the cut-off point of DAS28 at RRR-study entry was 2.225. Subsequently, one-way analysis of DAS28 at the primary end point by DAS28 at study entry, <2.225 versus between 2.225 and 3.2, was performed and the statistical difference of the two groups was sought by non-parametric Wilcoxon t test (***p<0.001). ESR, erythrocyte sedimentation rate.